

Decline of the Hemoglobin Adduct of 4-Aminobiphenyl during Withdrawal from Smoking¹

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ABSTRACT

The hemoglobin adduct of the human bladder carcinogen 4-aminobiphenyl (4ABP-Hb) declined in the blood of 34 smokers enrolled in a withdrawal program, from a mean of 120 ± 7 (SE) pg/g of hemoglobin at the start to a mean of 82 ± 6 pg/g after 3 weeks and a mean of 34 ± 5 pg/g among the 15 exsmokers who had not resumed smoking after 2 months. Although 4ABP-Hb declined faster than expected under the assumption that the human erythrocyte has a life span of 120 days, it persisted much longer than cotinine. Therefore, 4ABP-Hb may complement the use of cotinine as a marker of exposure to tobacco smoke. The strength of the within-person association of 4ABP-Hb with smoking, coupled with the weakness of the between-person association (correlation coefficient, 0.33), is evidence that between-person variation in modifying factors is substantial. Study of the modifiers of 4ABP-Hb levels may help elucidate the etiology of human susceptibility to aromatic amine-induced bladder cancer.

INTRODUCTION

The aromatic amine 4ABP⁴ is a potent human bladder carcinogen (1, 2) and is present in tobacco smoke (3). Its carcinogenicity is believed to derive from its *N*-hydroxylation and subsequent hydrolysis yielding an electrophilic nitrenium ion that binds with DNA and other macromolecules (4, 5). We have shown in a rat model (6, 7) that the acid-labile sulfonamide adduct, formed by reaction of *N*-hydroxy-4-aminobiphenyl with a cysteine residue of hemoglobin, reflects average exposure to 4ABP over the life span of the rat erythrocyte. Using a sensitive gas chromatography-mass spectrometry assay (8), we have also shown that blood levels of 4ABP-Hb are 2 to 10 times higher in smokers than in nonsmokers (8-10). To assess the potential of the assay as a dosimeter for epidemiological studies of 4-aminobiphenyl, we undertook a validation study involving 34 smokers enrolled in a withdrawal program. Our hypotheses were: (a) adduct levels would decline during withdrawal; (b) the rate of decline would correspond to the rate of replacement of erythrocytes after a life span of 120 days; (c) differences among the initial adduct levels of smokers before they began the program would be explained by differences in the number and type of cigarettes smoked; and (d) 4ABP-Hb would be as good a dosimeter of tobacco smoke exposure as plasma cotinine, a metabolite of nicotine now widely used as a biochemical marker of smoking.

MATERIALS AND METHODS

Volunteers were recruited from smoking withdrawal classes conducted by The Stop Smoking Clinic (Danvers, MA) at 7 suburban

hospitals in Eastern Massachusetts. Their ages ranged from 23 to 78 years, with a mean of 44 years. Fifty-five % of them were women. The initial blood sample was collected from 75 subjects at the beginning of a 2-week reduction period. Tar and nicotine intake was reduced 75% during the first week and the remaining 25% during the second week, inasmuch as the program required the participants not only to smoke steadily fewer cigarettes per day but also to switch to brands with progressively lower tar and nicotine levels. The second blood sample was collected 3 weeks after the first, and the third blood sample was collected 6 to 8 weeks after the second.

Blood samples were drawn into 10-ml EDTA vacutainers and refrigerated overnight. Erythrocytes were separated from plasma, washed in saline, and frozen. Plasma was frozen and sent to the American Health Foundation, Valhalla, NY, for determination of cotinine by a modified radioimmunoassay (11, 12). A plasma cotinine level below 10 ng/ml was taken as an indicator of successful withdrawal (13). After all blood samples had been collected from a subject, the cells were thawed, processed, and assayed simultaneously to prevent confounding by any day-to-day variation in laboratory methods.

The procedures of sample preparation and analysis have been described in detail elsewhere (8). In brief, hemoglobin was isolated from RBC and purified by dialysis. The dialysate was spiked with 1 ng of an internal standard (4'-fluoro-4-aminobiphenyl) and then hydrolyzed to release free 4ABP. The amines were extracted into purified hexane and then derivatized to form the corresponding pentafluoropropionamides. These were quantified using capillary gas chromatography with detection by negative-ion chemical ionization mass spectrometry.

The precision of the assay was quantified by measuring the coefficient of variation after splitting two 100-ml blood samples into 8 and 7 aliquots and by comparing duplicate blood samples collected 15 to 48 h apart from 15 people (8).

Differences and associations were examined both graphically and statistically. Mean differences and correlation coefficients were calculated with 95% confidence intervals. Differences between the observed and expected percent declines in excess adduct as a function of time were tested by the one-sample *t* test.

The observed percent decline in excess adduct due to smoking was defined as the decline in the difference between the observed adduct level and the final adduct level attained by the exsmokers. The percent decline in the excess between the first and second blood samples was calculated, and likewise between the first and third samples, using three different estimates of the final attained level: the mean adduct level in 9 subjects who had not resumed smoking 4 months after withdrawal (33 pg/g of hemoglobin); and the upper and lower limits of the 95% confidence interval about the mean (23 and 44 pg/g of hemoglobin).

The expected percent decline in excess at time, *t*, was computed with the assumption of sudden cessation of exposure and uniform life expectancy, *L*, for all erythrocytes. Under these assumptions, the decline in excess adduct is expected to be quadratic, not linear, because two processes of linear decline take place simultaneously and interact. Not only is there a linear fall in the number of cells ever exposed to the excess 4ABP from smoking but there is also a linear fall in the average cumulative exposure of the remaining exposed cells. This is because the first erythrocytes to be replaced are the oldest, the cells with the greatest cumulative exposure to aromatic amine. After the first 1% of cells are replaced, 99% of the remaining cells were ever exposed to adducts from tobacco smoke and, among them, the average excess of adduct per cell is 99% the original average. Therefore, the total excess adduct remaining in the blood is $99\% \times 99\% = 98\%$. In general, the remaining fraction of the original excess of adduct equals $(1 - t/L)^2$, where *t/L* is time since exposure cessation, expressed as a fraction of

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⁴ The abbreviations used are: 4ABP, 4-aminobiphenyl; 4ABP-Hb, 4-aminobiphenyl-hemoglobin.

the erythrocyte lifespan. (See Ref. 14 for the derivation.) The percent decline in excess is $100\% - (1 - t/L)^2 \times 100\%$. Various expected percent declines in excess 4ABP-Hb were computed assuming values for L ranging from 60 to 120 days.

The expected percent of decline in excess adduct was subtracted from the difference between the observed percent decline and one of the three estimates of the final level of 4ABP-Hb attained (the mean, or the upper or lower bound of the confidence interval). The mean difference between observed and expected percent decline was divided by its standard error and interpreted as a t statistic, for which a two-sided P value was determined.

RESULTS

Only 34 of the 75 initial participants had successfully withdrawn from smoking by the third week and were still willing to participate in the study. By 9–11 weeks, this number had dropped to 15.

All but 2 of the 34 who had withdrawn by the third week showed reductions in the level of 4ABP-Hb (Fig. 1). All 15 who remained exsmokers after 9–11 weeks showed continued reductions.

The mean values for cotinine and 4ABP-Hb over time are shown in Table 1. The mean difference over the first 3 weeks was 38 pg/g of hemoglobin (95% confidence interval, 29 to 47 pg/g). The mean difference by 9–11 weeks was 75 pg/g of hemoglobin (95% confidence interval, 60 to 89 pg/g).

The observed percent decline in excess adduct was significantly greater than expected, assuming an erythrocyte life span of 120 days, for all comparisons of observed *versus* expected, except when the calculations assumed the background level was the lower bound of the confidence interval for the mean attained level in exsmokers (23 pg/g). Even with such a low estimate of the attained (or normal background) level, the percent decline in excess over the first 3 weeks was calculated to be 40% (95% confidence interval, 31–49%) compared with an expected decline of 32%. By 9–11 weeks, it was 90% (95% confidence interval, 83–98%) compared with an expected decline of 79%. Differences between observed and expected percent declines, under various assumptions about erythrocyte life span and final attained levels in exsmokers, are shown in Table 2.

The association between level of adduct at time of enrollment in The Stop Smoking Clinic and reported prior cigarette consumption was weak (Fig. 2). The correlation coefficient was

0.33 (95% confidence interval, 0.11–0.52) and this was mainly attributable to the lack of heavy smoking subjects with low 4ABP-Hb values. Variation in the type of cigarettes smoked did not appear to explain the weakness of this association. Some heavy smokers of high tar brands had only moderately elevated 4ABP-Hb and some light smokers of low tar brands had relatively high levels of 4ABP-Hb.

There was also little association between 4ABP-Hb and plasma cotinine levels (Fig. 3); the correlation coefficient was 0.23 (95% confidence interval, 0.01–0.43). The correlation coefficient for plasma cotinine levels and reported cigarette consumption was 0.30 (95% confidence interval, 0.08–0.50).

DISCUSSION

Our first hypothesis was corroborated. Our data showed unequivocally that smoking withdrawal results in lower levels of 4ABP adducts with hemoglobin. Since these adducts are thought to form by the same mechanism by which 4ABP adducts with DNA are formed and 4ABP is a known human bladder carcinogen believed to initiate the disease through its reaction with DNA, our study demonstrated that smoking causes the yield of a potentially carcinogenic reaction to increase 2–10 times above its background yield in nonsmokers. Cessation of smoking results in a decline to yields found in nonsmokers.

Our second hypothesis was not corroborated. The average rate of decline of 4ABP-Hb levels exceeded the expected rate, despite the fact that the smokers did not quit suddenly but cut down both the number and strength of their cigarettes gradually over the first 2 weeks. Presumably the rate of decline would have been only faster if withdrawal had been more precipitous. One possible explanation is that the life span of erythrocytes is markedly reduced in smokers. This is conceivable but unlikely to account fully for our results. Assuming 33 pg/g as the final attained level in exsmokers, the observed decline over the first 3 weeks was most compatible with an erythrocyte life span of 80 days, while the decline over 9–11 weeks was most compatible with a life span of less than 60 days. Assuming 23 pg/g as the final level, the observed declines at 3 and 9–11 weeks were most compatible with life span estimates of 95 and 100 days, respectively. A more likely explanation is that there is some degradation of 4ABP-Hb adduct over time.

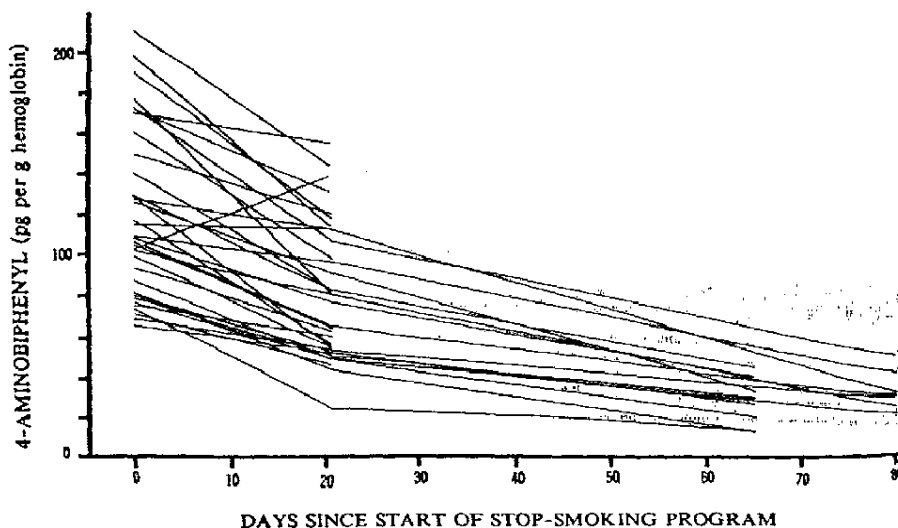


Fig. 1. Levels of 4-aminobiphenyl adducts with hemoglobin over time, among 34 participants in a smoking withdrawal program.

Table 1 Concentrations of plasma cotinine and hemoglobin adducts of 4-aminobiphenyl among smokers enrolled in a smoking withdrawal program

No. of subjects	Days since starting to quit	Cotinine (ng/ml)	4-Aminobiphenyl (pg/g of hemoglobin)
34	0	306 ± 26 ^a	120 ± 7
34	20-23	1.5 ± 0.5	82 ± 6
15	65-80	0.9 ± 0.2	34 ± 5
9	120-160	0 ± 0	33 ± 6

^a Mean ± SE.

Table 2 Differences between observed and expected percent declines in excess levels of 4ABP-Hb among smokers enrolled in a withdrawal program, using different assumptions about erythrocyte life span and mean level of adduct finally attained by the exsmokers

Time since start of program (days)	Assumed final level of 4ABP-Hb in exsmokers (pg/g)	Observed mean (±SE) of individual % declines in excess 4ABP-Hb	Difference between observed and expected % declines in excess 4ABP-Hb, under 4 different assumptions about erythrocyte life span			
			120 days	100 days	80 days	60 days
20-23	23 ^a	40 ± 5	8	2	-6	-18 ^b
	33 ^a	46 ± 5	14 ^b	8	1	-12 ^b
	44 ^a	56 ± 7	24 ^b	18 ^b	11 ^b	-2
65-80	23	90 ± 4	11 ^b	3	-6	-9 ^b
	33	107 ± 6	28 ^b	19 ^b	10	7
	44	132 ± 9	53 ^b	44 ^b	36 ^b	33 ^b

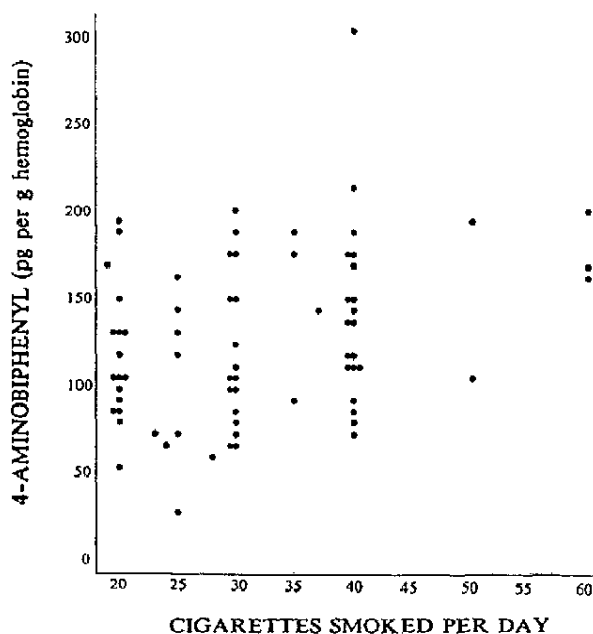
^a Mean and 95% confidence limits of 4ABP-Hb levels observed in 9 exsmokers more than 120 days after withdrawal.^b Two-sided *P* < 0.05.

Fig. 2. Relationship between levels of 4-aminobiphenyl adducts with hemoglobin at the start of a smoking withdrawal program, and daily consumption of cigarettes during the preceding month.

Our third and fourth hypotheses were not corroborated in these data. There was only a weak association between the level of 4ABP-Hb at the start of the withdrawal program and the daily consumption of cigarettes during the previous month. The correlation coefficient of 0.33 equal to that seen here was equal to that observed in another study (9). This correlation coefficient is no better than the correlation between cotinine level and number of cigarettes smoked.

Cotinine has the advantage of being detectable in "involun-

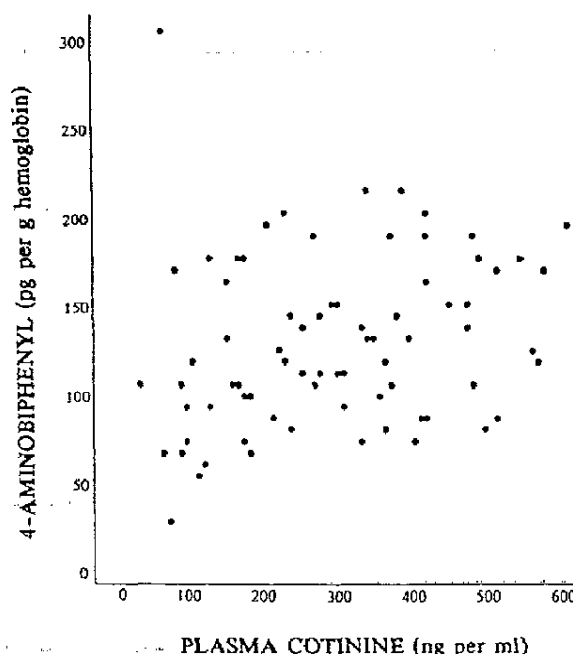


Fig. 3. Relationship between levels of 4-aminobiphenyl adducts with hemoglobin and plasma cotinine levels at the start of a smoking withdrawal program.

tary smokers" and undetectable among nonsmokers who are not passively exposed (15). By contrast, a substantial background level of exposure to 4ABP from unknown sources exists in nonsmokers and exsmokers. Another advantage of cotinine is its presence in urine, which can be sampled less invasively than blood. The chief limitation of cotinine is the time period it represents; it is an indicator of exposure only during the previous 2-4 days (16). 4ABP-Hb adduct levels evidently reflect average exposure during the past several weeks.

The utility of 4ABP-Hb as a dosimeter will grow as we gain understanding of the other sources of 4ABP and the factors that modify the rate of adduct formation with hemoglobin *in vivo*. The weakness of the between-person association of 4ABP and cigarette smoke intake, when compared with the striking associations within individuals, suggests that factors such as diet (17), acetylation phenotype (18, 19), or induction of enzymes by other ingredients of tobacco smoke (20) may obscure the relation between 4ABP-Hb and 4ABP intake. Investigation of the correlates of 4ABP-Hb levels among people who have the same intake of cigarette smoke may help identify such modifying factors. Statistical adjustment for these factors would reduce between-person noise in 4ABP-Hb levels, thus improving the utility of 4ABP-Hb as a dosimeter for 4ABP intake. Since the metabolism of 4ABP is believed representative of that of other aromatic amines, such as 2-naphthylamine (4), investigation of host factors that modify 4ABP-Hb levels could also help elucidate the etiology of human susceptibility to aromatic amine-induced bladder cancer.

Finally, it is worth mentioning that our study demonstrated the utility of using smokers in a withdrawal program as a population for studies of carcinogen metabolism in humans. Our indirect evidence that modifying factors obscure the relation between intake of cigarettes and 4ABP-Hb levels underscores the need to control for these factors. Until they are known, however, the best way to control for them is to use subjects as their own controls, *i.e.*, by studying people when they are exposed to the carcinogens and when they are not.

Standard experiments, however, are impossible with human subjects. It is unethical to give carcinogens to volunteers. By contrast, it is perfectly ethical to study people who decide to stop consuming carcinogens regularly. In fact, several subjects said they found the ordeal of withdrawal was made easier by the knowledge that they were contributing to a study. The main difficulty with this kind of research is the high percentage of subjects who fail to quit, necessitating collection of many more blood samples than can be used.

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